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Functional analysis of *Gossypium hirsutum cellulose synthase catalytic subunit 4* promoter in transgenic *Arabidopsis* and cotton tissues

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ABSTRACT

Gossypium hirsutum cellulose synthase catalytic subunit 4 (*GhCesA4*) plays an important role in cellulose biosynthesis during cotton fiber development. The transcript levels of *GhCesA4* are significantly up-regulated as secondary cell wall cellulose is produced in developing cotton fibers. To understand the molecular mechanisms involved in transcriptional regulation of *GhCesA4*, β-glucuronidase (GUS) activity regulated by a *GhCesA4* promoter (−2574/+56) or progressively deleted promoters were determined in both cotton tissues and transgenic *Arabidopsis*. The spatial regulation of *GhCesA4* expression was similar between cotton tissues and transgenic *Arabidopsis*. GUS activity regulated by the *GhCesA4* promoter (−2574/+56) was found in trichomes and root vascular tissues in both cotton and transgenic *Arabidopsis*. The −2574/−1824 region was responsible for up-regulation of *GhCesA4* expression in trichomes and root vascular tissues in transgenic *Arabidopsis*. The −1824/−1355 region negatively regulated *GhCesA4* expression in most *Arabidopsis* vascular tissues. For vascular expression in stems and leaves, the −898/−693 region was required. The −693/−320 region of the *GhCesA4* promoter was necessary for basal expression of *GhCesA4* in cotton roots as well as *Arabidopsis* roots. Exogenous phytohormonal treatments on transgenic *Arabidopsis* revealed that phytohormones may be involved in the differential regulation of *GhCesA4* during cotton fiber development.

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1. Introduction

Cellulose, the most abundant biopolymer in nature, organizes into microfibrils in plant cell walls, providing strength and flexibility to plant tissues. Cellulose is synthesized by a plasma membrane associated, multisubunit enzyme called cellulose synthase [1]. The first plant cellulose synthase catalytic subunits (CesAs) were identified by comparing cotton fiber ESTs with bacterial cellulose synthase [2]. Extensive searches for *CesA* genes and mutant phenotypes in a model plant, *Arabidopsis* revealed that at least 10 different *cellulose synthase catalytic subunits* (*AtCesAs*) exist [3]. Three genes, *AtCesA1*, *AtCesA3*, and *AtCesA6* are expressed during primary cell wall (PCW) biosynthesis in roots and hypocotyls [4–6]. Another set of three genes, *AtCesA4*, *AtCesA7*, and *AtCesA8* are expressed during secondary cell wall (SCW) biosynthesis in *Arabidopsis* xylem cells [7–9]. *GhCesA1* [2] and *GhCesA4* [10] isolated from cotton fibers are orthologs of *AtCesA8* [9] involved in SCW cellulose biosynthesis in *Arabidopsis* [11]. The sequence comparison of *GhCesA1* (U58283) and *GhCesA4* (AF413210) with two BACs containing homologous *GhCesA1* genes showed that *GhCesA1* and *GhCesA4* are homologous genes of the D and A subgenomes of allotetraploid *Gossypium hirsutum*, respectively [12]. Northern blot analyses showed that *GhCesA1*, *2*, and *4* are specifically expressed in fiber tissues [2,10]. During cotton fiber development, transcript levels of *GhCesA1*, *2*, and *4* are significantly up-regulated at the transition from PCW to SCW biosynthesis [2,10].

Cotton (*G. hirsutum* L.) fibers are unicellular trichomes that differentiate from epidermal cells of developing cotton ovules [13]. Cotton fiber development is divided into four overlapping stages, (1) initiation, (2) PCW biosynthesis for fiber elongation, (3) SCW biosynthesis for cellulose production, and (4) maturation [14]. Fiber initiation starts a day before up to a day or two after anthesis, and the initials enter into the elongation phase immediately. During the PCW stage, a thin PCW is deposited in elongating fibers and cotton fibers elongate up to 3–6 cm for 2–3 weeks. The SCW stage initiates approximately 14–16 days post-anthesis (DPA), overlapping the final PCW stage. Mature fibers exhibit thickened SCW composed of nearly pure cellulose. At the transition from PCW to SCW biosynthesis in cotton fiber, synthesis of other cell wall polymers ceases and the rate of cellulose synthesis in cotton fibers are estimated to increase nearly 100-fold *in vivo* [15].

Although most genes involved in fiber elongation and cellulose biosynthesis in developing cotton fibers are transcriptionally regulated [2,10,11,13–15], difficulties in regenerating transgenic cotton

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have impeded the study of transcriptional regulation of cotton fiber genes. To circumvent the lengthy and labor intensive tissue culture procedures for constructing multiple transgenic lines of cotton plants, most functional analyses of cotton promoters have been studied in transgenic tobacco or *Arabidopsis* [16–22]. Since cotton fibers are seed trichomes, numerous cotton fiber specific promoters were studied in leaf trichomes of heterologous transgenic plants despite the limited understanding of potential similarities for transcriptional regulation between seed trichomes and leaf trichomes. Analyses of cotton fiber specific promoters using heterologous transgenic plants led to the identification of MYB and L1 as promoter motifs for trichome specific expression [18], and an AT-rich motif for repressing gene expression in non-fiber tissues [20]. In spite of these advances, whether the developmental and transcriptional regulation of cotton genes can similarly occur in heterologous transgenic plants is unknown. Therefore, cotton fiber specific promoter motifs identified from heterologous transgenic plants must be further verified in cotton tissues.

Although spatial regulations of cotton promoters involved in PCW biosynthesis during fiber development have been extensively studied using transgenic tobacco or *Arabidopsis* [16–21], comparatively less is known about cotton promoter activity involved in SCW biosynthesis during fiber development. A recent promoter activity assay of *GhCesA4*, a gene involved in SCW biosynthesis of developing cotton fibers, showed that *GhCesA4* was preferentially expressed in vascular tissues and induced by a synthetic auxin, NAA when a GUS reporter regulated by a short version (−1407/+106) of the *GhCesA4* promoter named P1482 was analyzed in transgenic tobacco [22].

To understand transcriptional regulation of SCW cellulose biosynthesis in cotton fibers, our group has also studied *GhCesA4* promoter activity using a longer version (−2574/+56) of the *GhCesA4* promoter (AF413210) isolated from *G. hirsutum* DPL90 [10,13]. In our study, we evaluated *GhCesA4* promoter activity by monitoring GUS expression in cotton tissues as well as transgenic *Arabidopsis* transformed stably or transiently regulated by the *GhCesA4* promoter (−2574/+56) or progressively smaller promoters. Consistent with the results reported by Wu et al. [22], we found that GUS activity regulated by one of the progressively deleted *GhCesA4* promoters (−1.355/+56), a size similar to P1482 (−1407/+106), was mainly detected in vascular tissues in both cotton tissues and transgenic *Arabidopsis*. Furthermore, we report here that one upstream region (−1.824/−1355) of the *GhCesA4* promoter is involved in down-regulating *GhCesA4* expression in vascular tissues and another upstream region (−2574/−1824) is involved in up-regulating *GhCesA4* expression in trichomes and roots. For basal expression of *GhCesA4* in both transgenic *Arabidopsis* and cotton roots, one downstream region (−693/−320) was required. We also show that several phytohormones differentially regulated *GhCesA4* promoter activity in various tissues at different developmental stages of transgenic *Arabidopsis*. In contrast to the previous report [22], our study shows by using the longer version of *GhCesA4* promoter (−2574/+56) that *GhCesA4* promoter activity was down-regulated by NAA in transgenic *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

Cotton plants (*G. hirsutum* L. TM-1) were grown in the field at the USDA, ARS, Southern Regional Research Center, New Orleans. Developing bolls were collected by 9 am at 4-day intervals from 8 through 24 DPA and fibers were immediately harvested and frozen in liquid nitrogen. Fully grown leaves (15 cm in diameter), expanding young leaves (5 cm in diameter), hypocotyls and roots were harvested from 1 or 6-week-old plants grown in a greenhouse at 25–32 °C. All tissues were frozen in liquid nitrogen, and stored at −80 °C. *Arabidopsis* plants were grown at 23 °C under 16 h light/8 h dark photoperiod.

2.2. Functional analysis of GhCesA4 promoter (−2574/+56) in various cotton tissues

The longest *GhCesA4* promoter (−2574/+56) fused to a *GUS* reporter was constructed using the pCAMBIA vector 1391z [23] and named pCes1. For transient expression, one micron gold particles coated with the pCes1 construct were bombarded into various cotton tissues using a Biolistic Particle Delivery System (1000/He) according to the described method [10]. For stable transformation, the pCes1 construct was introduced into *Agrobacterium rhizogenes* ATCC #15834 and subsequently inoculated on cotyledon leaves of *G. hirsutum*. Cotton hairy roots were developed and cultured according to the described method [24]. Transgenic cotton roots containing pCes1 were screened on HRIM medium [25] with 50 mg/L hygromycin. Localization of GUS activity was carried out using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) [26]. To prevent diffusion of the GUS product during staining, 0.5 mM potassium ferri/ferrocyanide was added to the histochemical staining buffer.

2.3. RNA extraction and quantitative RT-PCR

Total RNA was extracted from cotton fibers and other tissues at different developmental stages using a Plant Total RNA Kit and DNase I (Sigma, St. Louis, MO). First strand complementary DNA was synthesized using 1 μg of total RNA by priming with random hexamers at 48 °C for 30 min followed by inactivation of MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) at 95° C for 10 min. Quantitative reverse transcription-PCR (qRT-PCR) was performed using the SYBR® Green PCR Master Mix with a specific primer set for *GhCesA4* (5′-CCTTGCCTTGGACTACCCTGTA-3′/5′-CTTTCTTGCAAAGTCGGCTGTT-3′; amplicon size, 109 bp). The transcript levels of *GhCesA4* were normalized with respect to *18S ribosomal RNA* (5′-CGTCCCTGCCCTTTGTACA-3′/5′-AACACTTCACCGGACCATTCA-3′; amplicon size, 63 bp). A total of six qRT-PCR reactions were performed at each time point for cotton tissues representing two biological replications and three technical replications. Statistical analyses and construction of graphs were performed using Prism version 3.00 software (GraphPad Software, Inc., San Diego, CA).

2.4. Promoter deletion assays in transgenic Arabidopsis and cotton tissues

The progressively shorter versions of the *GhCesA4* promoter were PCR-amplified, cloned into pCR-XL-TOPO vector (Invitrogen, Carlsbad, CA) and confirmed by DNA sequencing. All *GhCesA4* promoters were fused to the *GUS* reporter using the pCAMBIA vector 1391z and named orderly according to the length of the promoter sequence: pCes1 (−2574/+56), pCes2 (−1824/+56), pCes3 (−1355/+56), pCes4 (−898/+56), pCes5 (−693/+56), pCes6 (−320/+56), and pCes7 (−174/+56). The promoterless pCAMBIA 1391z was used as a negative control, pCes8. All constructs were introduced into *Arabidopsis* through *Agrobacterium tumefaciens* strain GV3101 using a floral dip method [27] or into cotton hairy roots through *Agrobacterium rhizogenes* ATCC #15834 using a tissue culture method [25]. Transgenic *Arabidopsis* and transgenic cotton hairy roots were selected on media containing 50 mg/mL of hygromycin. Average quantitative GUS activity controlled by each 5′ deleted *GhCesA4* promoter was determined from soluble pro-

tein from cauline leaves of multiple transgenic *Arabidopsis* lines (4–15 lines). Both quantitative and histochemical GUS assays was carried out according to the described method [26]. Images of histochemically stained tissues were taken with an Olympus SZX stereomicroscope with an Olympus DP11 digital camera. Image composites were constructed using Adobe Photoshop software.

2.5. Exogenous phytohormonal treatments

Transgenic *Arabidopsis* seedlings of six days after germination (6 DAG) were transferred and incubated on 0.5× MS medium containing 10 μM 1-naphthaleneacetic acid (NAA), 50 μM abscisic acid (ABA), 10 nM brassinolide (BL), 5 μM gibberellic acid (GA_3), or 5 μM kinetin for 1, or 3 days. Mock-treated controls were incubated under the same conditions with the buffer alone.

3. Results

3.1. Sequence analysis of GhCesA4 promoter

The upstream sequences from the translational start codon of *GhCesA4* consist of 2681 nucleotides (Fig. 1). Computational analysis [28] showed that the putative transcriptional start site of *GhCesA4* is located 107 nucleotides upstream from the translational start codon and the transcriptional start site is marked as +1 (Fig. 1). By using two algorithms of PLACE [29] and PlantCARE [30], a number of putative promoter motifs were identified within the *GhCesA4* promoter composed of 2574 nucleotides. A putative TATA box is located in the region −25/−30, and a putative CAAT box is present in the region −70/−75 (Fig. 1). There are a number of phytohormone responsive motifs such as auxin response factor binding motifs (AuxRR-Core [31], ARFAT [32], and TGA box [33]), gibberellin response motifs (P box [34] and TATC box [35]), ABA response motifs (MYB core [36], MYB1AT [37], RY [38,39] and ABRE [40]) (Fig. 1 and Table 1). In addition, the *GhCesA4* promoter contains other putative motifs for tissue specific expression. They are tracheary element regulating *cis*-elements (TERE) involved in secondary wall formation and programmed cell death [41], a BS1 motif involved in vascular specific expression [42], a combination of an L1 box and a MYB motif involved in trichome specific expression [18], and AT-rich promoter sequences involved in repressing gene expression in non-fiber tissues [20] (Fig. 1 and Table 1).

3.2. Analysis of GhCesA4 promoter in cotton tissues

We first tested if the longest *GhCesA4*promoter (−2574/+56) in the pCes1 construct is functional in cotton tissues. The pCes1 constructed by fusing the *GhCesA4* promoter (−2574/+56) with a *GUS* reporter were transformed transiently or stably into various cotton tissues and the promoter activity was histochemically analyzed (Fig. 2).

When pCes1 was transiently transformed in cotton tissues, the blue color representing GUS activity in both leaf trichomes (Fig. 2A) and hypocotyls trichomes (Fig. 2B) showed that the *GhCesA4* promoter (−2574/+56) was functionally active in cotton trichomes. Despite *GhCesA4* was reported to be a fiber specific gene by Northern blot analysis [10], *GhCesA4* promoter was functionally active in non-trichome tissues such as hypocotyls (Fig. 2C) and leaves (Fig. 2D) transiently transformed with pCes1. In the epidermal tissues from hypocotyls and leaves (Fig. 2B–D), gossypol (brown spots) as well as GUS activity (blue spots) were detected. When pCes1 was expressed in stably transformed cotton hairy roots, GUS activity was detected in most elongating zone of cotton roots except in the root cap (Fig. 2E). No GUS activity was found in cotton hairy roots stably transformed with a promoterless pCes8 construct (Fig. 2F).

3.3. Fiber preferential expression of GhCesA4 in cotton plant

We reexamined *GhCesA4* expression pattern quantitatively using qRT-PCR, a more sensitive technique than Northern blot analyses because non-quantitative histochemical assay (Fig. 2) showed *GhCesA4* expression in both trichome and non-trichome tissues. The *GhCesA4* primer set for qRT-PCR was designed to amplify *GhCesA4*, but not other available cotton *GhCesA* genes whose sequences are deposited in the GenBank database. Consistent with the results from Northern blot analysis [10], the qRT-PCR result showed that *GhCesA4* was mainly expressed during the SCW biosynthesis stage of fiber development. *GhCesA4* transcript levels were low during PCW biosynthesis when rapid fiber elongation occurs (8–12 DPA), markedly increased at the onset of SCW biosynthesis (14–16 DPA), and remained in high levels during SCW biosynthesis (20–24 DPA) (Fig. 3A).

When *GhCesA4* levels were compared among various cotton tissues by qRT-PCR (Fig. 3B), low but detectable levels of *GhCesA4* were found among non-fiber tissues although *GhCesA4* was most abundantly expressed in 20 DPA cotton fibers (F-20). Among non-fiber tissues, higher *GhCesA4* transcript levels were detected in actively elongating tissues like 1-week-old hypocotyls (S-1) and roots (R-1) than in mature tissues such as 6-week-old stems (S-6) and roots (R-6). Low but detectable levels of *GhCesA4* were also detected in expanded leaves (EL) (Fig. 3B). Consistent with the histochemical assays of the *GhCesA4* promoter (−2574/+56) (Fig. 2), the qRT-PCR results suggested that basal levels of *GhCesA4* were expressed in non-fiber tissues. Thus, *GhCesA4* was preferentially, but not specifically, expressed during the SCW biosynthesis stage in developing cotton fibers (Fig. 3A and B).

3.4. Quantitative assay of 5′ deletions of the GhCesA4 promoter in transgenic Arabidopsis

To characterize the cotton *GhCesA4* promoter, progressive 5′ deletions of the *GhCesA4* promoter, pCes2 (−1824/+56), pCes3 (−1355/+56), pCes4 (−898/+56), pCes5 (−693/+56), pCes6 (−320/+56), and pCes7 (−174/+56) in addition to pCes1 (−2574/+56) containing the longest *GhCesA4* promoter, were fused to the *GUS* gene and transformed into *Arabidopsis* (Fig. 4). Average quantitative GUS activity performed with soluble protein from cauline leaves of multiple transgenic *Arabidopsis* lines showed that the highest GUS activity was in transgenic plants regulated by the longest *GhCesA4* promoter in the pCes1 construct (Fig. 4: pCes1). After the first 5′ deletion from −2574 (pCes1) to −1824 (pCes2), GUS activity sharply decreased to nearly undetectable levels in transgenic plants (Fig. 4: pCes2). After the 5′ deletion from −1824 (pCes2) to −1356 or −898 (pCes3 and pCes4), recognizable GUS activity of the pCes3 construct was detected (Fig. 4: pCes3) and GUS activity of the pCes4 construct showed a second peak (Fig. 4: pCes4). With the shorter constructs (pCes5–pCes7), little GUS activity was detected (Fig. 4: pCes5–pCes7). These results imply that two promoter regions (−2574/−1825 and −1355/−694) may be involved in up-regulation of *GhCesA4*, and one region (−1824/−1356) may repress *GhCesA4* in leaf tissues of transgenic *Arabidopsis*.

3.5. Histochemical assay of 5′ deletions of GhCesA4 promoter in transgenic Arabidopsis

To localize the tissues and organs responsible for *GUS* expression by *GhCesA4* promoters in transgenic *Arabidopsis*, histochemical analyses were performed (Fig. 5). The GUS activity regulated by

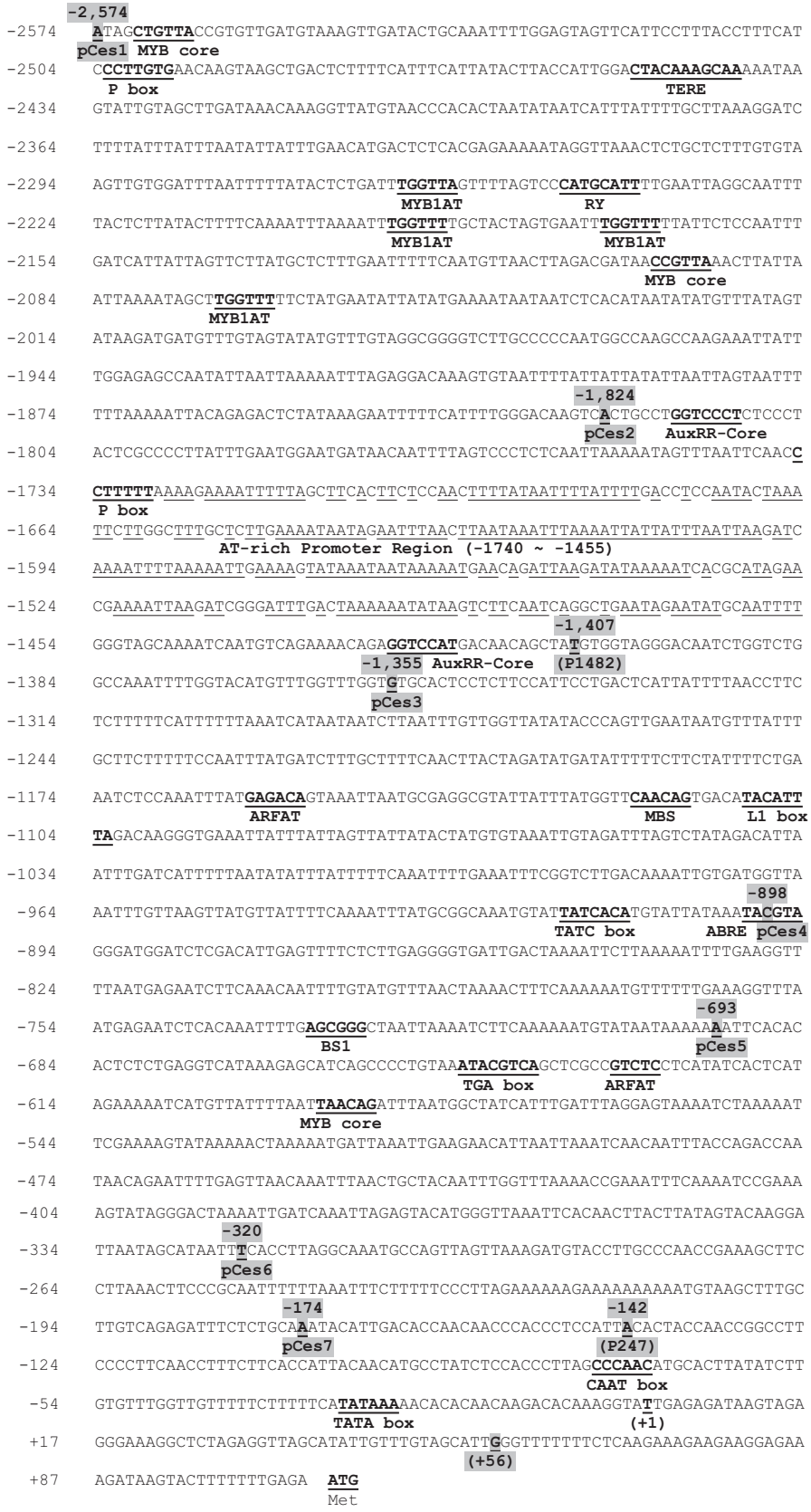

Fig. 1. *GhCesA4* promoter sequence. The putative transcription start site is denoted +1. The putative promoter motif sequences are underlined. The 5′ and 3′ sequences of *GhCesA4* promoter deletion constructs (pCes1, 2, 3, 4, 5, 6, and 7) are highlighted in gray. The 5′ sequences of the longest P1482 (−1407/+106) and the shortest P247 (−142/+106) used in Wu et al. [22] are also highlighted in gray.

Table 1
Putative motifs of the *GhCesA4* promoter.

Motifs ID	Positions	Sequence	Functions	References
MYB core	−2570, −2099, −633	CNGTTR	MYB binding site	[36]
P box	−2503, −1735	CCTTttt/CCTTgtg	Gibberellin-responsiveness	[34,35]
TERE	−2451	CTACAAAGCAA	SCW formation and programmed cell death	[41]
MYB1AT	−2264, −2194–2174, −2072	WAACCA	MYB binding site, ABA signaling	[37]
RY	−2248	CATGcatt	Seed-specific regulation, ABA signaling	[38,39]
AuxRR-Core	−1817, −1425	GGTCcat/GGTCcct	Auxin responsiveness	[31]
AT-rich promoter region	−1728 ~ −1455	AT rich sequences	Negative elements in non-fiber tissues	[20]
ARFAT	−1159, −592	TGTCTC/GAGAC	Auxin response factor binding site	[32]
MBS	−1121	CAACtg	MYB binding site	[40]
L1 box	−1110	TAAATGYA	Trichome specific expression	[18]
TATC box	−918	TATCaca	Gibberellins responsiveness	[34,35]
ABRE	−900	cctACGTatt	Abscisic acid responsiveness	[40]
BS1	−733	AGCGGG	Vascular specific expression	[42]
TGA box	−648	TGACgtat	Auxin-responsiveness	[33]

the longest *GhCesA4* promoter (pCes1) was strongly detected in all trichomes of rosette leaves (Fig. 5A), stems (Fig. 5B), and cauline leaves (Fig. 5C). In addition to trichomes, pCes1 showed GUS activity in all root tissues, especially strong GUS activity was observed in the root tip consisting of the columella and lateral root cap where active growth occurs (Fig. 5D). After the first 5′ deletion from −2574 (pCes1) to −1824 (pCes2), almost no GUS activity was detected in most tissues of transgenic *Arabidopsis* (Fig. 5E–H). Very low but detectable GUS activity from pCes2 was found only in a few trichomes localized at the tip of emerging rosette leaves (Fig. 5E), but most trichomes located in most area of rosette leaves, stems and cauline leaves showed no detectable GUS activity (Fig. 5E–G). In addition, very low and sporadic GUS activity was found in the vascular tissue of primary roots (Fig. 5H). After the 5′ deletion from −1824 (pCes2) to −1356 or −898 (pCes3 and pCes4), GUS activity in trichomes was not changed, but GUS activity was present in all tested vascular tissues of stems, leaves, and roots (Fig. 5I–P). After the 5′ deletion from −898 (pCes4) to −693 (pCes5), the GUS localization patterns in pCes5 were almost identical to those in pCes2 (−1824/+56) showing no GUS activity in vascular tissues in most tissues except roots (Fig. 5Q–T). There was no detectable GUS expression in transgenic plants transformed by the constructs of pCes6 (−320/+56) and pCes7 (−174/+56) (Fig. 5U–BB).

The histochemical images of cauline leaves from each construct (Fig. 5C, G, K, O, S, W, and AA) were consistent with the quantitative GUS assays performed with soluble protein from cauline leaves (Fig. 4). The results of histochemical and quantitative assays showed that the strong GUS activity identified from the cauline leaf of pCes1 (Fig. 4: pCes1) was localized in trichomes (Fig. 5C), and the mild GUS activity identified from the cauline leaf of pCes3 and pCes4 (Fig. 4: pCes3 and pCes4) was localized in vascular tissues (Fig. 5K and O).

Overall, the histochemical assays (Fig. 5) showed that two *GhCesA4* promoter regions were responsible for trichome expression in transgenic *Arabidopsis*. One promoter region (−2574/−1824) containing one TERE and several MYB motifs (Table 1 and Fig. 4) was required for trichome specific expression because strong GUS activity was specifically found in trichomes of pCes1 transformed tissues (Fig. 5A), but the other constructs did not exhibit this activity (Fig. 5E, I, M, Q, U, and Y). The other promoter region (−693/−320) was also required for basal expression of *GhCesA4* within trichomes because very low but detectable GUS activity in the trichomes located at the tip of developing leaves was found from pCes2 to pCes5 (Fig. 5E, I, M, and Q), but not in pCes6 and pCes7 (Fig. 5U and Y). Similarly, two different promoter regions may be involved in vascular expression in different tissues from transgenic *Arabidopsis*. One region (−898/693) containing a BS1 motif (Table 1 and Fig. 4) was responsible for vascular tissues of stems and leaves because GUS activity were specifically detected in vascular tissues of stems and leaves from pCes3 and pCes4 (Fig. 5J, K, N, and O). The other promoter region (−693/−320) was also required for basal expression of *GhCesA4* in the vascular tissues of primary roots from transgenic *Arabidopsis* (Fig. 5T).

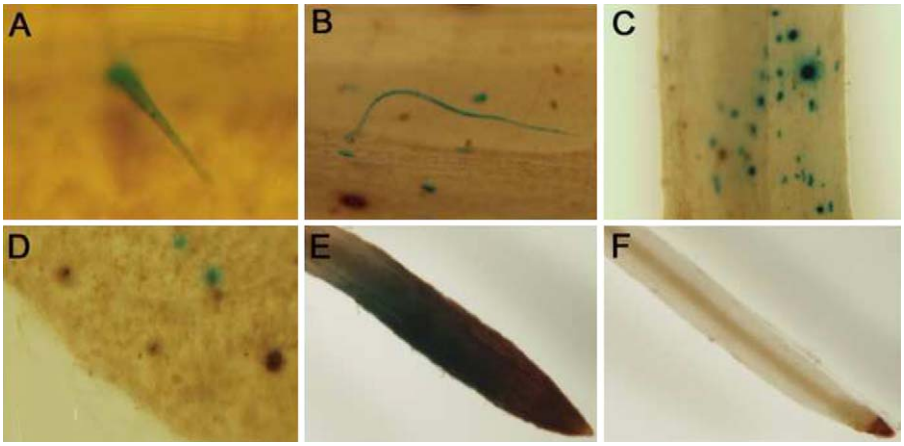

Fig. 2. Histochemical analyses of GUS activity regulated by *GhCesA4* promoter (−2574/+56) in the pCes1construct in various cotton tissues. pCes1 was expressed transiently in leaf trichome (A), hypocotyls trichomes (B), hypocotyls (C), and leaf (D). pCes1 was also expressed in stably transformed cotton hairy roots (E). As a negative control, the promoterless pCAMBIA 1391z (pCes8) was transformed into cotton hairy roots (F). Localization of GUS activity was carried out using X-Gluc.

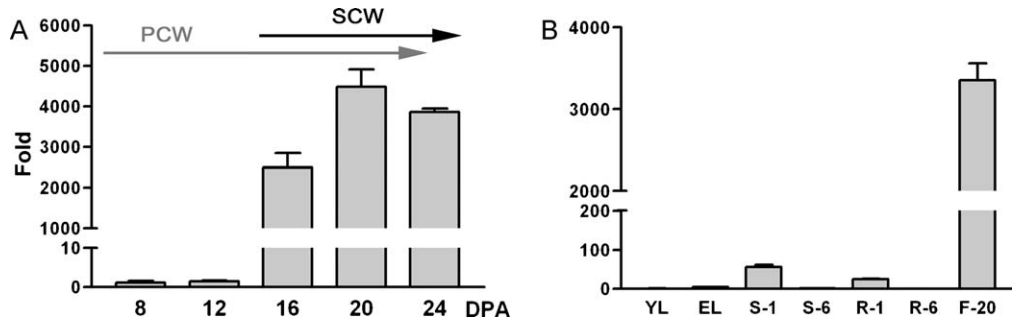

Fig. 3. Expression profiles of *GhCesA4* determined by qRT-PCR. The transcript levels of *GhCesA4* in cotton tissues were normalized with respect to the transcript level of *18S ribosomal RNA*. (A) Developmental *GhCesA4* expression in developing cotton fibers. Primary cell wall (PCW); secondary cell wall (SCW) biosynthesis stage. (B) Preferential *GhCesA4* expression in various cotton tissues. *GhCesA4* levels were compared among young leaves (YL), fully expanded leaves (EL), 1-week old hypocotyls (S-1), 6-week-old stems (S-6), 1-week-old roots (R-1), 6-week-old roots (R-6), and 20-DPA fibers (F-20).

3.6. Histochemical assay of 5′ deletions of GhCesA4 promoter in cotton tissues

To further characterize the *GhCesA4* promoters in cotton tissues, each promoter deletion construct of Fig. 4 was expressed in stably transformed cotton hairy roots. Histochemical analyses of the deletion assay of *GhCesA4* promoter in stably transformed cotton hairy roots (Fig. 6) showed the similar GUS expression patterns with those in stably transformed transgenic *Arabidopsis* roots (Fig. 5). Consistent with the strong GUS activity of pCes1 in the roots from transgenic *Arabidopsis* (Fig. 5D), GUS activity of pCes1 was also detected in most cotton root tissues except in the root cap (Fig. 6A). GUS activity of pCes2–pCes5 were mainly detected in vascular tissues of elongating cotton hairy roots (Fig. 6B–E) as detected in vascular tissues of transgenic *Arabidopsis* roots (Fig. 5H, L, P, and T). Interestingly, GUS activity of pCes4 and pCes5 was found in the root cap (Fig. 6D and E). GUS activity driven by pCes6 and pCes7 was not found in cotton hairy roots (Fig. 6F and G) like no GUS activity in transgenic *Arabidopsis* roots (Fig. 5X and BB). Based on the *GhCesA4* expression profiles in developing cotton fibers (Fig. 3A) and various cotton tissues (Fig. 3B), low and basal levels of *GhCesA4* were expressed in cotton roots. Therefore, the −693/−320 region containing two auxin response motifs (TGA and ARFAT) and a MYB binding motif may be important for basal expression of *GhCesA4* in cotton roots (Fig. 6). The same −693/−320 region was required for basal expression in the vascular tissues of primary roots and trichomes from transgenic *Arabidopsis* (Fig. 5).

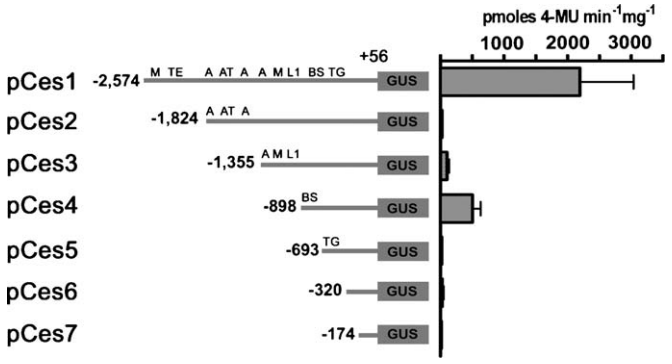

Fig. 4. Schematic diagram and quantitative GUS assays of 5′ *GhCesA4* promoter deletion constructs. Putative promoter motifs of *GhCesA4* promoter are labeled as M (MYB binding motif for trichome expression) TE (TERE motif involved in SCW formation and programmed cell death), A (AuxRR-Core and ARF binding motifs for auxin responsiveness), AT (AT-rich promoter region for repression in nonfiber tissues), L1 (L1 box for ovule and trichome expression), BS (BS1 motif for vascular expression), and TG (TGA motif for auxin responsiveness). A quantitative GUS assay was performed with cauline leaves from transgenic *Arabidopsis* using 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate.

3.7. Phytohormonal regulations of GhCesA4 in transgenic Arabidopsis

To monitor how *GhCesA4* levels are regulated by phytohormones, we first treated pCes1 transgenic *Arabidopsis* with a number of exogenous phytohormones, and quantitatively measured the GUS activity in response to phytohormone treatment. Using whole pCes1 seedlings consisting of diverse tissues with different developmental stages in this assay resulted in no statistically significant effect of phytohormone treatment on GUS activity, regardless of phytohormone concentrations or duration of incubation period (data not shown). We suspected that a lack of phytohormone effect on GUS activity with whole seedlings might be caused by the differential regulation of *GhCesA4* in diverse tissues and different developmental stages. The histochemical images of Fig. S1 confirmed that NAA regulated *GhCesA4* differentially in diverse tissues of transgenic *Arabidopsis*. NAA increased GUS activity in hypocotyls and lateral roots of pCes1 seedlings, whereas NAA decreased GUS activity in primary roots and cotyledons (Fig. S1). Therefore, we decided to determine phytohormone effects using primary roots from transgenic *Arabidopsis* because *Arabidopsis* xylem has been recently suggested to be a good model for studying secondary wall cellulose synthesis in cotton fiber [11]. To avoid complications from potential developmental regulation of genes within primary roots, we focused on the maturing zone of *Arabidopsis* primary roots.

When pCes1 plants (6 DAG) were pre-incubated with 10 μM NAA, GUS activity of the primary root was significantly decreased within 1 day and almost disappeared in 3 days (Fig. 7C and D) compared with that in control roots (Fig. 7A and B). GUS activity slightly decreased with the treatment of 50 μM ABA for 1 and 3 days (Fig. 7E and F). In contrast, GUS activity increased after a 1 day incubation with 10 nM brassinolide (Fig. 7G), 5 μM gibberellic acid (Fig. 7I), or 5 μM kinetin (Fig. 7K). The 3-day treatments of brassinolide (Fig. 7H) and kinetin (Fig. 7L) continued to increase GUS activity comparing with GUS activity in control plants (Fig. 7B), whereas there was little difference among treatments between gibberellic acid (Fig. 7J) and the control (Fig. 7B) for 3 days. As a result, brassinolide and kinetin up-regulated *GhCesA4*, but NAA down-regulated *GhCesA4* in the maturating zone of primary roots from transgenic *Arabidopsis*.

4. Discussion

In this study, we first analyzed *GhCesA4* promoter activity quantitatively and histochemically with cauline leaves from transgenic *Arabidopsis*. Since *GhCesA4* was developmentally regulated during leaf vascular development in transgenic *Arabidopsis* and each rosette leaf has different developmental stage according to the order of appearance from the leaf primordium, we used two sim-

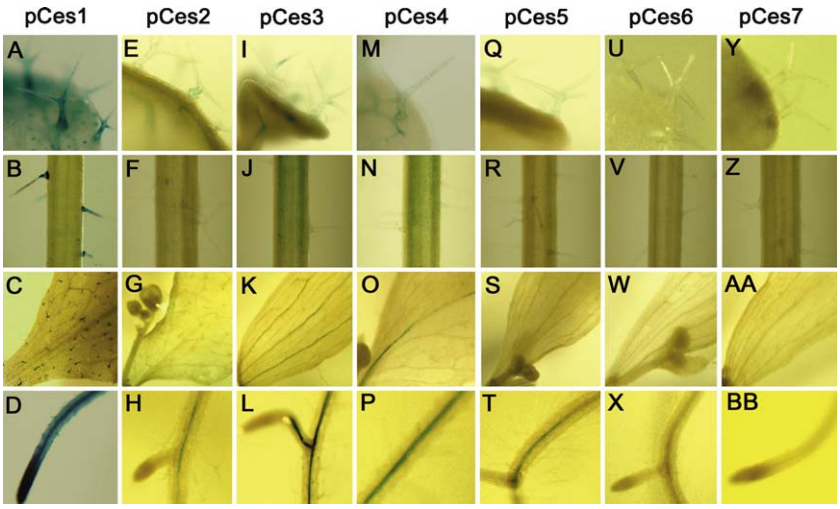

Fig. 5. Histochemical analyses of transgenic *Arabidopsis* transformed by 5′ *GhCesA4* promoter deletion constructs. GUS activity in transgenic *Arabidopsis* transformed by pCes1 (A–D), pCes2 (E–H), pCes3 (I–L), pCes4 (M–P), pCes5 (Q–T), pCes6 (U–X), or pCes7 (Y–BB) was histochemically determined in the tip of rosette leaves containing trichomes (the 1st row: A, E, I, M, Q, U, and Y), stems (the 2nd row: B, F, J, N, R, V, and Z), cauline leaves (the 3rd row: C, G, K, O, S, W, and AA) and primary roots (the 4th row: D, H, L, P, T, X, and BB).

ilar sized (∼0.5 cm) cauline leaves at the same position from the stem for each transgenic line to perform both quantitative and histochemical assays. Consistent results from the quantitative assay of cauline leaves (Fig. 4) and the histochemical assays of cauline leaves (Fig. 5C, G, K, O, S, W, and AA) were obtained.

In addition, we histochemically analyzed *GhCesA4* promoter activity in cotton hairy roots as well as transgenic *Arabidopsis* roots. *A. rhizogenes*-induced hairy roots have recently become a molecular tool to study tissue-specific expression patterns of promoters and the subcellular localization of proteins in other plant systems [43]. Hairy roots are morphologically very similar in structure to wild-type roots, although hairy roots show a high incidence of lateral branching and grow in an agravitropic manner [43,44]. Promoter analyses tested in both hairy root and normal roots of transgenic plants showed an identical spatial expression pattern of *Enod40* promoter activity [45]. Similarly, tissue-specific expression patterns have been successfully determined with various promoters fused to GUS or GFP [46–49]. Moreover, hairy roots have been successfully used to determine the subcellular localization of proteins to various organelles [43,50–53]. Thus, we used cotton hairy roots to test *GhCesA4* promoter activity in vascular tissues. Our results showed similarities in the spatial regulation of *GhCesA4* expression between cotton hairy roots and transgenic *Arabidopsis* roots (Figs. 5 and 6).

GUS activity regulated by the longest *GhCesA4* promoter was detected in trichomes and vascular tissues of roots in both cotton and transgenic *Arabidopsis* (Figs. 2, 5, and 6). The results from the promoter deletion assay showed that the −2574/−1824 region containing six MYB motifs is important for the up-regulation or tissue specific expression of *GhCesA4* expression in trichomes (Fig. 5A, B, and C). Our results support the notion that MYB motifs are sufficient for trichome specific expression [54]. The −1824/−1355 region containing high AT-rich sequences was involved in repressing *GhCesA4* expression in vascular tissues of transgenic *Arabidopsis* (Figs. 4 and 5). A similar high AT-rich element (84 bp) of the cotton lipid transfer protein (*FSltp4*) promoter was involved in repressing the expression of *FSltp4* in non-fiber tissues [20]. For vascular specific expression, the −898/−693 region containing a BS1 motif (−733) was required for expression in stems and leaves (Fig. 5J, K, N, and O). The BS1 motif confers vascular expression of the *cinnamoyl-CoA reductase* promoter from *Eucalyptus gunnii* [42]. The −693/−320 region containing two auxin response motifs (TGA and ARFAT) and a MYB binding motif was necessary for basal expression of *GhCesA4* in *Arabidopsis* trichomes localized at the leaf tips (Fig. 5). Auxin is primarily produced in hydathodes located at the leave tips [55,56]. The same −693/−320 region was also required for basal expression of *GhCesA4* in both cotton roots and *Arabidopsis* roots (Figs. 5 and 6).

Recently, another research group has studied *GhCesA4* promoter activity in transgenic tobacco with a shorter version of the *GhCesA4* promoter (−1407/+106) sharing 97% sequence identity to our *GhCesA4* promoter [22]. The longest *GhCesA4* promoter (−1407/+106) named P1482 by the other research group is almost similar to one of our deletion constructs, namely pCes3 (−1355/+56). GUS activity regulated by both P1482 in transgenic tobacco and pCes3 in transgenic *Arabidopsis* was found in vascular tissues of stems. Despite similar results between P1482 and pCes3, there are some discrepancies between the two assays. NAA had an opposite effect on *GhCesA4* expression in the two assays. In transgenic tobacco seedlings, NAA up-regulated GUS activity regulated by P1482 the most (1.86-fold) among the tested phytohormones [22]. In contrast, our results showed that NAA down-regulated *GhCesA4* expression the most. GUS activity in the primary roots of transgenic *Arabidopsis* transformed by pCes1 was

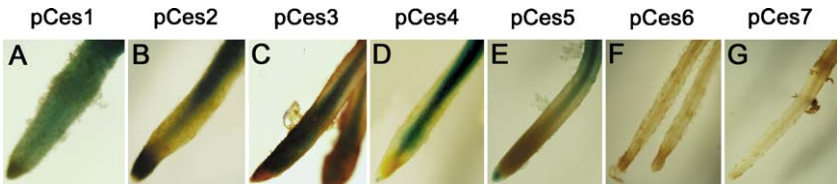

Fig. 6. Histochemical analyses of cotton hairy roots transformed by 5′ *GhCesA4* promoter deletion constructs. GUS activity in cotton hairy roots stably transformed by pCes1 (A), pCes2 (B), pCes3 (C), pCes4 (D), pCes5 (E), pCes6 (F), or pCes7 (G) was histochemically determined.

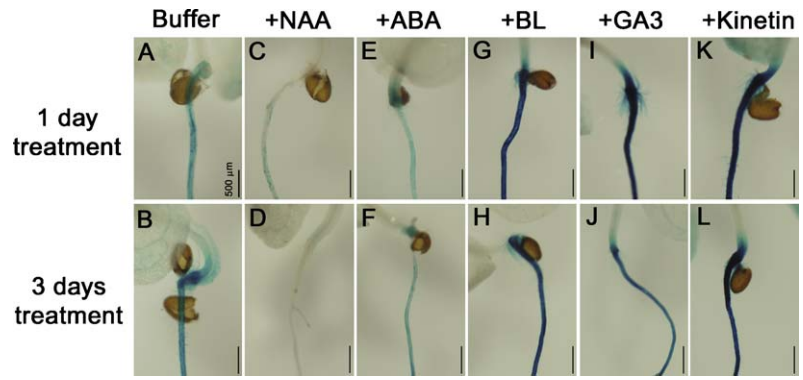

Fig. 7. Phytohormonal regulation of *GhCesA4* in primary roots of transgenic *Arabidopsis*. Transgenic *Arabidopsis* seedlings (pCes1, 6 DAG) were incubated with the buffer alone (A and B) as a control, 10 μM NAA (C and D), 50 μM ABA (E and F), 10 nM brassinolide (G and H), 5 μM GA_3 (I and J) or 5 μM kinetin (K and L) for 1 day (the 1st row: A, C, E, G, I, and K), or 3 days (the 2nd row: B, D, F, H, J, and L).

noticeably reduced after 1-day incubation with 10 μM NAA, and almost disappeared by 3-day incubation with 10 μM NAA (Fig. 7C and D). Although GUS is known as a stable reporter protein, a recent study shows that strongly expressed blue GUS activity in *Arabidopsis* whole plants transformed by a stress responding promoter fused to GUS decreased significantly as a result of stress treatments within 8 h [57]. Our results were consistent with a recent report that NAA suppressed secondary wall cellulose synthesis and enhanced elongation of cultured cotton fiber when compared with IAA (indole-3-acetic acid) [58]. Although IAA is the predominant natural auxin in plants, we intentionally used NAA, a synthetic auxin because NAA is more stable than IAA and can be diffused into plant cells in contrast IAA uptake requires protein carriers [55,58]. The length of *GhCesA4* promoter between pCes1 (−2574/+56) and P1482 (−1407/+106) may contribute to the opposite NAA effects on *GhCesA4* expression because P1482, a shorter version of *GhCesA4* (−1407/+106) does not contain a number of phytohormone response motifs, including two auxin response motifs that are located upstream (−2574/−1407) of the P1482 promoter. In addition, kinetin did not show much effect on P1482 expression in transgenic tobacco seedlings (~1.4-fold increase), whereas in our experiments kinetin as well as brassinolide up-regulated pCes1 significantly in the primary roots of transgenic *Arabidopsis* (Fig. 7). In other plant systems like *Zinnia* and *Arabidopsis* xylem, auxin, cytokinin, and brassinosteroids are involved in the regulation of SCW biosynthesis [59,60]. Based on the results that NAA regulated *GhCesA4* expression differentially and developmentally in various tissues (Fig. S1), we suspect that whole seedlings with complex tissues and different developmental stages may not be an appropriate system for studying phytohormone effects on *GhCesA4* expression. It is likely that the differences between heterologous plant systems may contribute to different spatial regulation of *GhCesA4* expression. In transgenic tobacco, two short *GhCesA4* promoters containing the −321/+106 region (P424) or the −142/+106 region (P247) were required for vascular expression [22]. In our study, two short *GhCesA4* promoters containing the −320/+56 region (pCes6) or the −174/+56 region (pCes7) were not necessary for vascular expression in transgenic *Arabidopsis* (Fig. 5U–BB). Using cotton hairy roots, we confirmed that the two shortest promoters (pCes6 and pCes7) did not support expression in vascular tissues (Fig. 6F and G).

Despite the limits of using heterologous systems, transgenic *Arabidopsis* and tobacco have been used for studying cotton fiber genes and promoters due to the challenges in constructing multiple transgenic cotton lines. In this study, we showed that GUS activity driven by *GhCesA4* promoter was developmentally and spatially regulated in transgenic *Arabidopsis* tissues (Figs. 3 and 5). We also showed that phytohormonal regulations of the *GhCesA4* promoter in transgenic *Arabidopsis* were various among different tissues at specific developmental stages (Fig. 7 and Fig. S1). Therefore, studies of cotton fiber genes and promoters using heterologous systems require extra caution for identifying appropriate tissues with the correct developmental stage from heterologous plants. Based on the results from *GhCesA4* promoter assay in transgenic *Arabidopsis* and *in silico* promoter motif analyses, we concluded that primary roots of transgenic *Arabidopsis* can be used to optimize the conditions for conducting *GhCesA4* promoter assays in cotton. Consistent with our results that exogenous NAA treatments down-regulated *GhCesA4* expression in primary roots of transgenic *Arabidopsis* (Fig. 7C and D), exogenous NAA treatments of cultured cotton fibers also down-regulated *GhCesA1* and *GhCesA2* that are involved in SCW cellulose biosynthesis during cotton fiber development [58].

In summary, we demonstrated that one upstream region (−2574/−1824) of the *GhCesA4* promoter was involved in up-regulating *GhCesA4* expression in trichomes and root vascular tissues, the −1824/−1355 region of the *GhCesA4* promoter was involved in down-regulating *GhCesA4* expression in vascular tissues, and the −693/−320 region was necessary for basal expression of *GhCesA4* in the cotton hairy roots as well as *Arabidopsis* roots. We suggest that several phytohormones may be potentially involved in the differential regulation of *GhCesA4* expression during fiber development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.10.003.

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